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REPLACEMENT SHEET

Appln No.: 10/658,355

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Applicant(s): Rene Gantier, et. al

RATIONAL DIRECTED PROTEIN EVOLUTION USING TWO-DIMENSIONAL RATIONAL MUTAGENESIS SCANNING

Identification of is-HITs

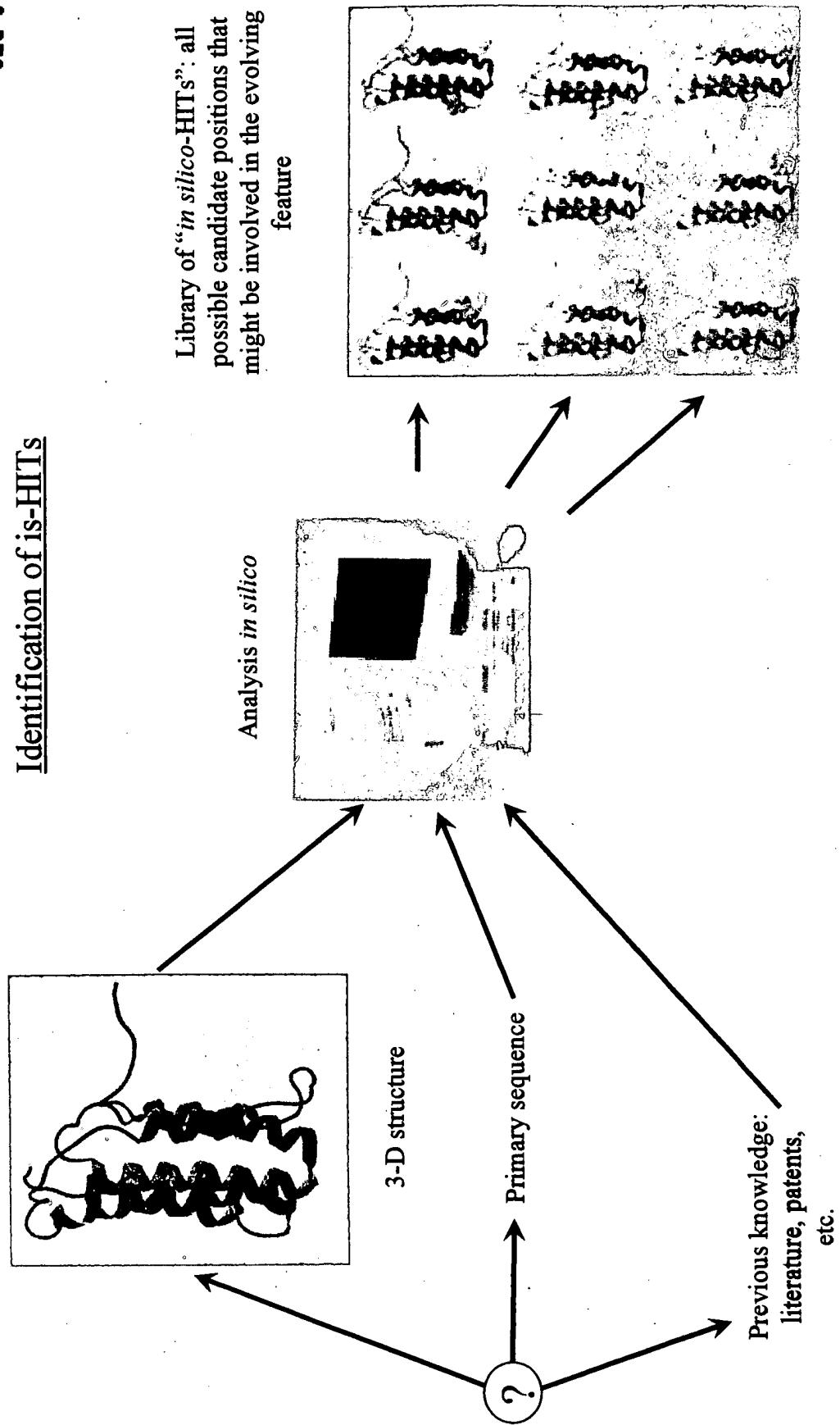


FIG. 1A

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Applicant(s): Rene Gantier, et. al

RATIONAL DIRECTED PROTEIN EVOLUTION USING TWO-DIMENSIONAL RATIONAL MUTAGENESIS SCANNING

Identification of LEAD candidates

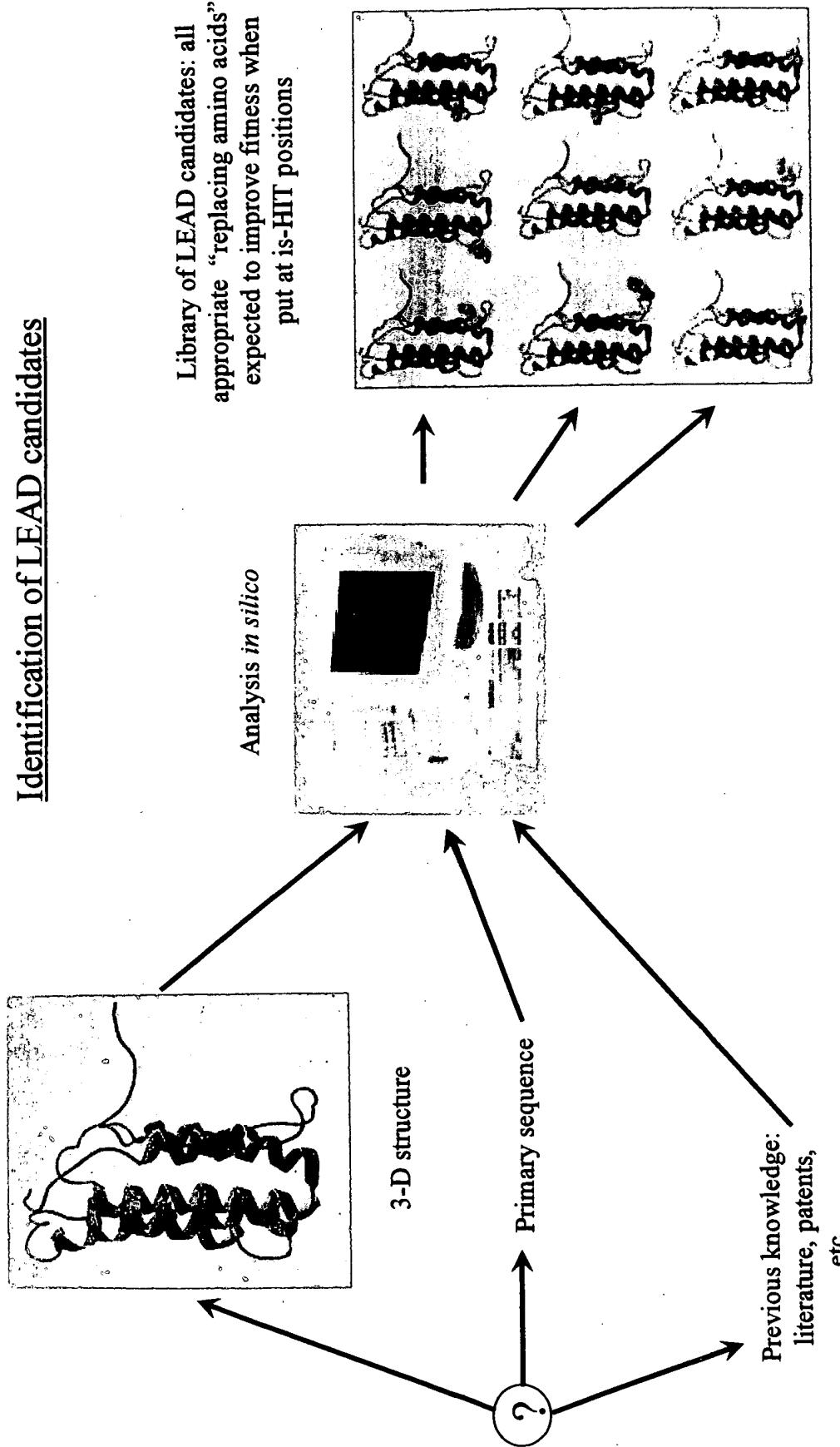


FIG. 1B

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Applicant(s): Rene Gantier, et. al

RATIONAL DIRECTED PROTEIN EVOLUTION USING TWO-DIMENSIONAL RATIONAL MUTAGENESIS SCANNING

Identification of LEADS : the optimized sequences at the is-HIT positions

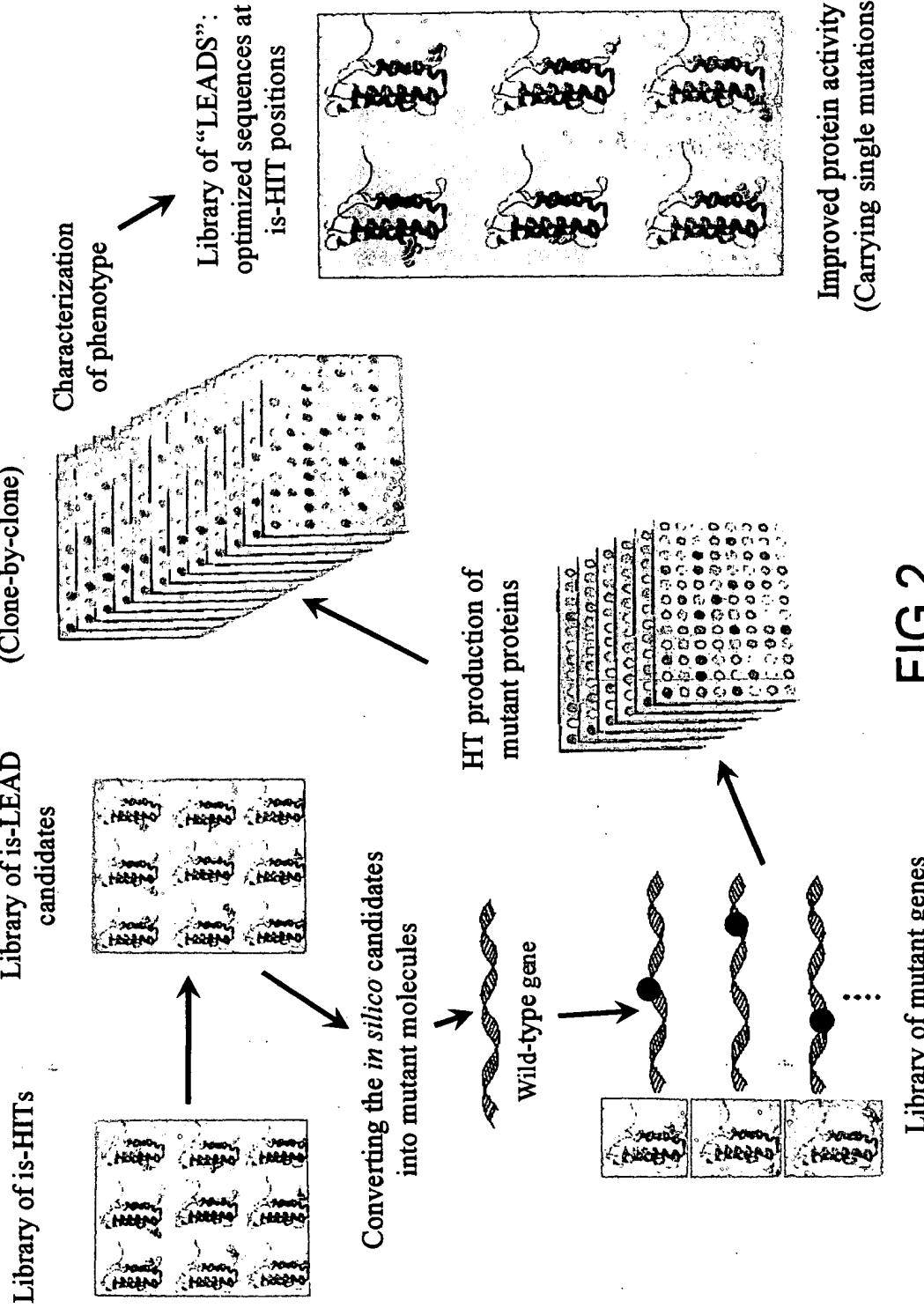


FIG.2

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RATIONAL DIRECTED PROTEIN EVOLUTION USING TWO-DIMENSIONAL RATIONAL MUTAGENESIS SCANNING

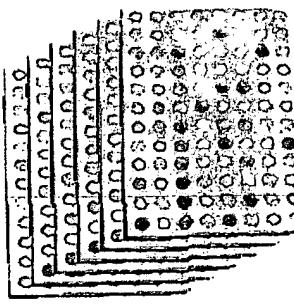
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Identification of SUPERLEADs

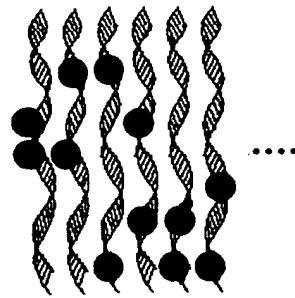
Library of "LEADS"



HT production of mutant proteins



Library of mutants (Combined LEADs)



LEAD sequences

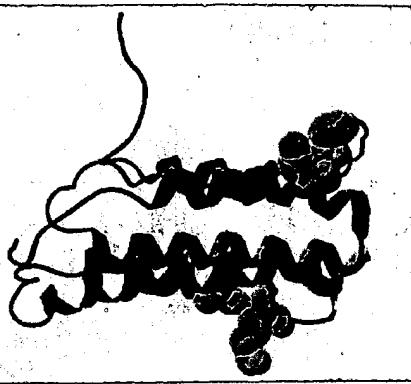


FIG. 3A

Improved protein activity: carrying
multiple LEAD mutations

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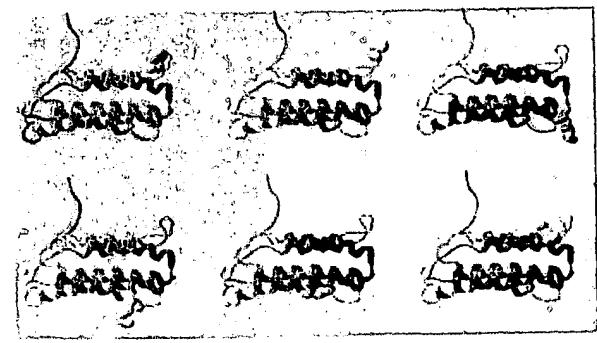
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Applicant(s): Rene Gantier, et. al

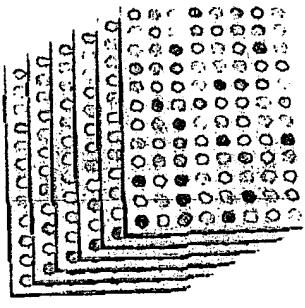
RATIONAL DIRECTED PROTEIN EVOLUTION USING TWO-DIMENSIONAL RATIONAL MUTAGENESIS SCANNING

Redesign of proteins

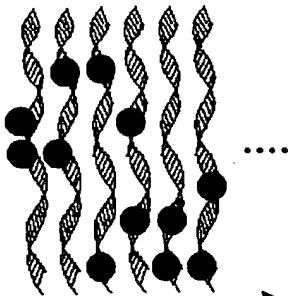
In silico library of
“pseudo wild-types”



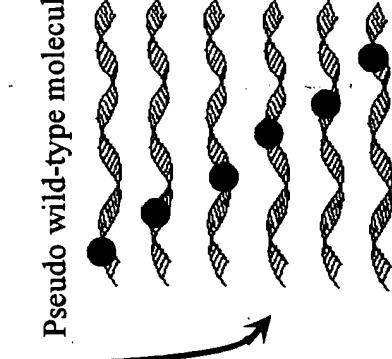
HT production
of mutant
proteins



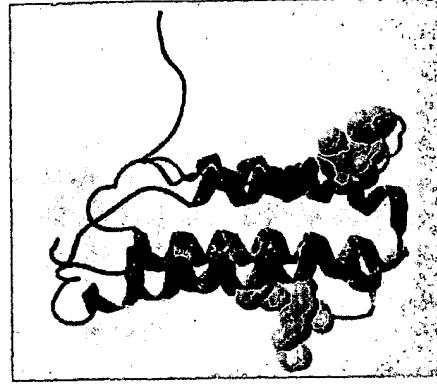
Library of mutants
(Combined pseudo wild-type sequences)



Pseudo wild-type molecules



Characterization of phenotype



Identification of redesigned pseudo wild-type
proteins (Comparable fitness but different
sequence compared to the wild-type)

FIG. 3B

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RATIONAL DIRECTED PROTEIN EVOLUTION USING TWO-DIMENSIONAL RATIONAL MUTAGENESIS SCANNING

“Additive Directional Mutagenesis” (ADM)

CONSTRUCT	Mutation 1	Mutation 2	Mutation 3	Mutation 4	Mutation 5	Mutation 6	Mutation 7	Mutation 8	Mutation 9
Mutation 1	Mutation 2								
	Mutation 2	Mutation 3							
	Mutation 2	Mutation 3	Mutation 4						
	Mutation 2	Mutation 3	Mutation 4	Mutation 5					
	Mutation 2	Mutation 3	Mutation 4	Mutation 5	Mutation 6				
	Mutation 2	Mutation 3	Mutation 4	Mutation 5	Mutation 6	Mutation 7			
	Mutation 2	Mutation 3	Mutation 4	Mutation 5	Mutation 6	Mutation 7	Mutation 8		
	Mutation 2	Mutation 3	Mutation 4	Mutation 5	Mutation 6	Mutation 7	Mutation 8	Mutation 9	
	Mutation 2	Mutation 3	Mutation 4	Mutation 5	Mutation 6	Mutation 7	Mutation 8	Mutation 9	Mutation 10
Mutation 2		Mutation 3							
	Mutation 3		Mutation 4						
	Mutation 3	Mutation 4	Mutation 5						
	Mutation 3	Mutation 4	Mutation 5	Mutation 6					
	Mutation 3	Mutation 4	Mutation 5	Mutation 6	Mutation 7				
	Mutation 3	Mutation 4	Mutation 5	Mutation 6	Mutation 7	Mutation 8			
	Mutation 3	Mutation 4	Mutation 5	Mutation 6	Mutation 7	Mutation 8	Mutation 9		
	Mutation 3	Mutation 4	Mutation 5	Mutation 6	Mutation 7	Mutation 8	Mutation 9	Mutation 10	
Mutation 3		Mutation 4							
	Mutation 4		Mutation 5						
	Mutation 4	Mutation 5	Mutation 6						
	Mutation 4	Mutation 5	Mutation 6	Mutation 7					
	Mutation 4	Mutation 5	Mutation 6	Mutation 7	Mutation 8				
	Mutation 4	Mutation 5	Mutation 6	Mutation 7	Mutation 8	Mutation 9			
	Mutation 4	Mutation 5	Mutation 6	Mutation 7	Mutation 8	Mutation 9	Mutation 10		
Mutation 4		Mutation 5							
	Mutation 5	Mutation 6							
	Mutation 5	Mutation 6	Mutation 7						
	Mutation 5	Mutation 6	Mutation 7	Mutation 8					
	Mutation 5	Mutation 6	Mutation 7	Mutation 8	Mutation 9				
	Mutation 5	Mutation 6	Mutation 7	Mutation 8	Mutation 9	Mutation 10			
Mutation 5		Mutation 6							
	Mutation 6	Mutation 7							
	Mutation 6	Mutation 7	Mutation 8						
	Mutation 6	Mutation 7	Mutation 8	Mutation 9					
	Mutation 6	Mutation 7	Mutation 8	Mutation 9	Mutation 10				
Mutation 6		Mutation 7							
	Mutation 7	Mutation 8							
	Mutation 7	Mutation 8	Mutation 9						
	Mutation 7	Mutation 8	Mutation 9	Mutation 10					
	Mutation 7	Mutation 8	Mutation 9	Mutation 10					
Mutation 7		Mutation 8							
	Mutation 8	Mutation 9							
	Mutation 8	Mutation 9	Mutation 10						
	Mutation 8	Mutation 9	Mutation 10						
Mutation 8		Mutation 9							
	Mutation 9	Mutation 10							
Mutation 9		Mutation 10							

FIG.4

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Applicant(s): Rene Gantier, et. al

RATIONAL DIRECTED PROTEIN EVOLUTION USING TWO-DIMENSIONAL RATIONAL MUTAGENESIS SCANNING

LEADs and SuperLEADs obtained for the Rep protein

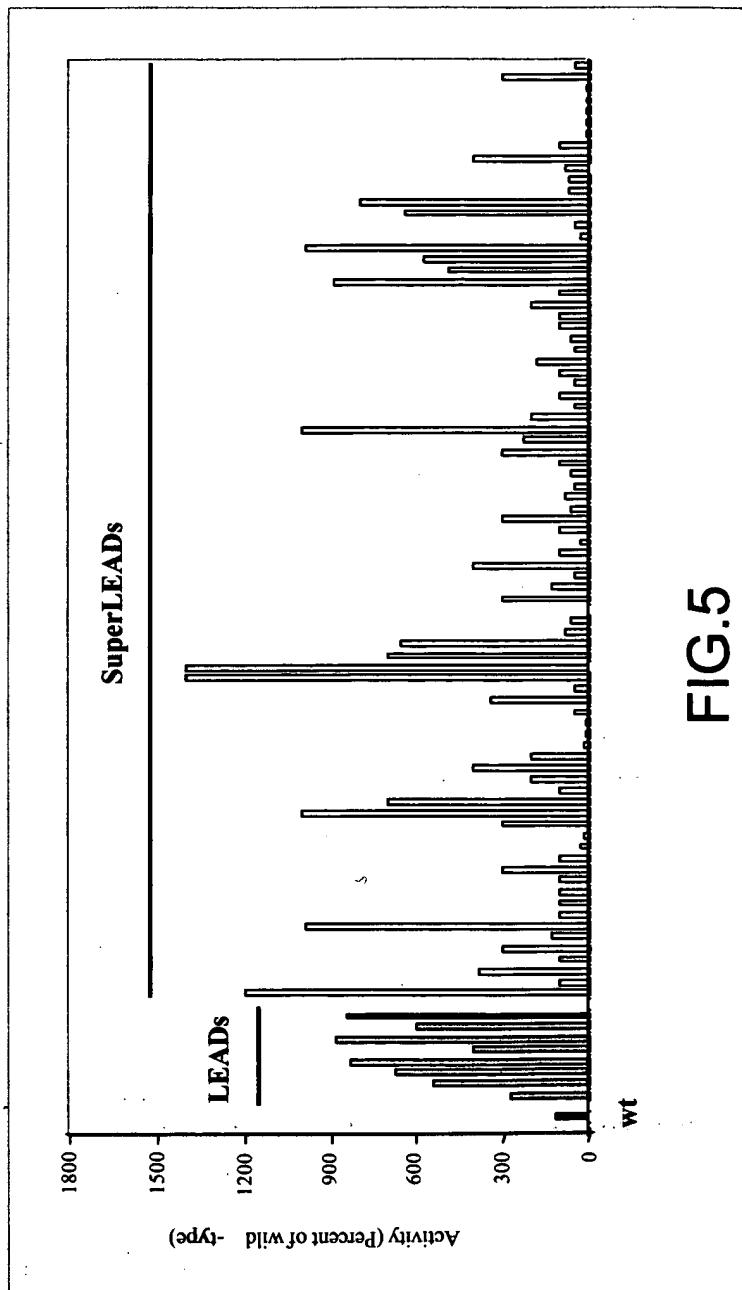


FIG.5

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RATIONAL DIRECTED PROTEIN EVOLUTION USING TWO-DIMENSIONAL RATIONAL MUTAGENESIS SCANNING

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Amino acid sequence of human mature IFN α -2b

IFN α -2b	1	10	20	30	40	50	
	•	•	•	•	•	•	
	<u>C</u> D <u>L</u> P<u>Q</u><u>T</u><u>H</u><u>S</u>L<u>G</u><u>S</u>R<u>R</u><u>T</u>L<u>M</u><u>L</u><u>A</u><u>Q</u>M<u>R</u><u>R</u><u>I</u>S<u>L</u>F<u>S</u><u>C</u><u>L</u>K<u>D</u>R<u>H</u><u>D</u><u>F</u><u>G</u><u>F</u>P<u>Q</u><u>E</u><u>E</u><u>F</u><u>G</u><u>N</u>Q<u>F</u>Q<u>K</u>A						
	51	60	70	80	90	100	
	•	•	•	•	•	•	
IFN α -2b		E <u>T</u> <u>I</u> <u>P</u> <u>V</u> <u>L</u> <u>H</u> <u>E</u> <u>M</u> <u>I</u> <u>Q</u> <u>Q</u> <u>I</u> <u>E</u> <u>N</u> <u>L</u> <u>F</u> <u>S</u> <u>T</u> K <u>D</u> <u>S</u> <u>S</u> <u>A</u> <u>A</u> <u>W</u> <u>D</u> <u>E</u> <u>T</u> <u>L</u> <u>D</u> <u>K</u> <u>F</u> <u>Y</u> <u>T</u> <u>E</u> <u>L</u> <u>Y</u> <u>Q</u> <u>Q</u> <u>L</u> <u>N</u> <u>D</u> <u>L</u> <u>E</u> <u>A</u> <u>C</u> <u>V</u> <u>I</u>					
	101	110	120	130	140	150	
	•	•	•	•	•	•	
IFN α -2b		<u>Q</u> <u>G</u> <u>V</u> <u>G</u> <u>V</u> <u>T</u> <u>E</u> <u>T</u> <u>P</u> <u>I</u> <u>M</u> <u>K</u> <u>E</u> <u>D</u> <u>S</u> <u>I</u> <u>I</u> <u>L</u> <u>A</u> <u>V</u> <u>R</u> <u>K</u> <u>Y</u> <u>F</u> <u>O</u> <u>R</u> <u>I</u> <u>T</u> <u>L</u> <u>K</u> <u>E</u> <u>K</u> <u>Y</u> <u>S</u> <u>P</u> <u>C</u> <u>A</u> <u>W</u> <u>E</u> <u>V</u> <u>V</u> <u>R</u> <u>A</u> <u>E</u> <u>I</u> <u>M</u> <u>R</u> <u>S</u>					
	151	160					
	•	•					
IFN α -2b		<u>F</u> <u>S</u> <u>L</u> <u>S</u> <u>T</u> <u>N</u> <u>L</u> <u>Q</u> <u>E</u> <u>S</u> <u>L</u> <u>R</u> <u>S</u> <u>K</u> <u>E</u>					

FIG.6A

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RATIONAL DIRECTED PROTEIN EVOLUTION USING TWO-DIMENSIONAL RATIONAL MUTAGENESIS SCANNING

Three dimensional structure of INF α -2b

showing candidate LEADS



FIG.6B

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Applicant(s): Rene Gantier, et. al

RATIONAL DIRECTED PROTEIN EVOLUTION USING TWO-DIMENSIONAL RATIONAL MUTAGENESIS SCANNING

The “Percent Accepted Mutation” (PAM250) matrix

	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
A	2	-2	0	0	-2	0	0	1	-1	-1	-2	-1	-1	-3	1	1	1	-6	-3	0
R	-2	6	0	-1	-4	1	-1	-3	2	-2	-3	3	0	-4	0	0	-1	2	-4	-2
N	0	0	2	2	-4	1	1	0	2	-2	-3	1	-2	-3	0	1	0	-4	-2	-2
D	0	-1	2	4	-5	2	3	1	1	-2	-4	0	-3	-6	-1	0	0	-7	-4	-2
C	-2	-4	-4	-5	12	-5	-5	-3	-3	-2	-6	-5	-5	-4	-3	0	-2	-8	0	-2
Q	0	1	1	2	-5	4	2	-1	3	-2	-2	1	-1	-5	0	-1	-1	-5	-4	-2
E	0	-1	1	3	-5	2	4	0	1	-2	-3	0	-2	-5	-1	0	0	-7	-4	-2
G	1	-3	0	1	-3	-1	0	5	-2	-3	-4	-2	-3	-5	0	1	0	-7	-5	-1
H	-1	2	2	1	-3	3	1	-2	6	-2	-2	0	-2	-2	0	-1	-1	-3	0	-2
I	-1	-2	-2	-2	-2	-2	-2	-3	-2	5	2	-2	2	1	-2	-1	0	-5	-1	4
L	-2	-3	-3	-4	-6	-2	-3	-4	-2	2	6	-3	4	2	-3	-3	-2	-2	-1	2
K	-1	3	1	0	-5	1	0	-2	0	-2	-3	5	0	-5	-1	0	0	-3	-4	-2
M	-1	0	-2	-3	-5	-1	-2	-3	-2	2	4	0	6	0	-2	-2	-1	-4	-2	2
F	-3	-4	-3	-6	-4	-5	-5	-5	-2	1	2	-5	0	9	-5	-3	-3	0	7	-1
P	1	0	0	-1	-3	0	-1	0	0	-2	-3	-1	-2	-5	6	1	0	-6	-5	-1
S	1	0	1	0	0	-1	0	1	-1	-1	-3	0	-2	-3	1	2	1	-2	-3	-1
T	1	-1	0	0	-2	-1	0	0	-1	0	-2	0	-1	-3	0	1	3	-5	-3	0
W	-6	2	-4	-7	-8	-5	-7	-7	-3	-5	-2	-3	-4	0	-6	-2	-5	17	0	-6
Y	-3	-4	-2	-4	0	-4	-4	-5	0	-1	-1	-4	-2	7	-5	-3	-3	0	10	-2
V	0	-2	-2	-2	-2	-2	-2	-1	-2	4	2	-2	2	-1	-1	-1	0	-6	-2	4

FIG.7

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Applicant(s): Rene Gantier, et. al

RATIONAL DIRECTED PROTEIN EVOLUTION USING TWO-DIMENSIONAL RATIONAL MUTAGENESIS SCANNING

Scores from PAM250, given to residue substitutions to protect
human INF α -2b against proteolysis

	R	D	E	L	K	M	F	P	W	I	Y
A	-2	0	0	-2	-1	-1	-3	1	-6	-3	
N	0	2	1	-3	1	-2	-3	0	-4	-2	
C	-4	-5	-5	-6	-5	-5	-4	-3	-8	0	
Q	1	2	2	-2	1	-1	-5	0	-5	-4	
G	-3	1	0	-4	-2	-3	-5	0	-7	-5	
H	2	1	1	-2	0	-2	-2	0	-3	0	
I	-2	-2	-2	2	-2	2	1	-2	-5	-1	
S	0	0	0	-3	0	-2	-3	1	-2	-3	
T	-1	0	0	-2	0	-1	-3	0	-5	-3	
V	-2	-2	-2	2	-2	2	-1	-1	-6	-2	

FIG.8

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Applicant(s): Rene Gantier, et. al

RATIONAL DIRECTED PROTEIN EVOLUTION USING TWO-DIMENSIONAL RATIONAL MUTAGENESIS SCANNING

Residue substitutions expected to allow the
creation of a disulfide bond



FIG.9A



FIG.9B

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Applicant(s): Rene Gantier, et. al

RATIONAL DIRECTED PROTEIN EVOLUTION USING TWO-DIMENSIONAL RATIONAL MUTAGENESIS SCANNING

Residue substitutions expected to destroy linking interactions

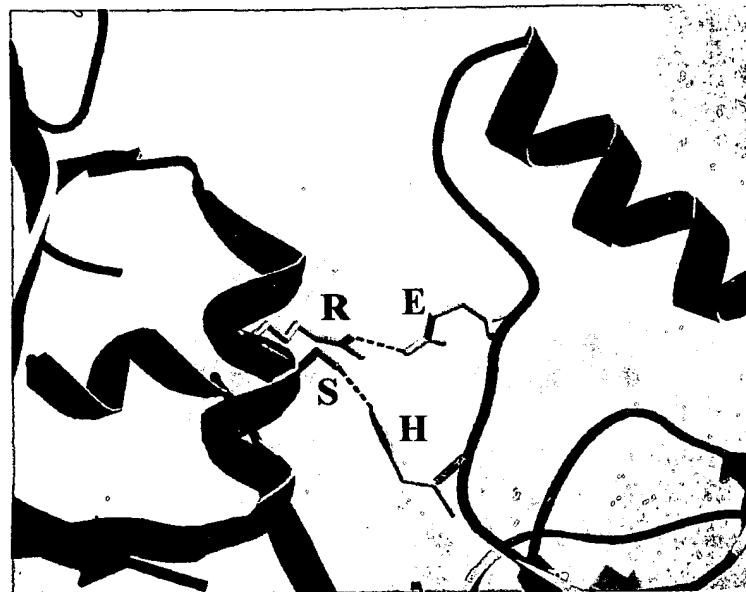


FIG.10A



FIG.10B

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Applicant(s): Rene Gantier, et. al

RATIONAL DIRECTED PROTEIN EVOLUTION USING TWO-DIMENSIONAL RATIONAL MUTAGENESIS SCANNING

Tri-dimensional model of an amphipathic polypeptide

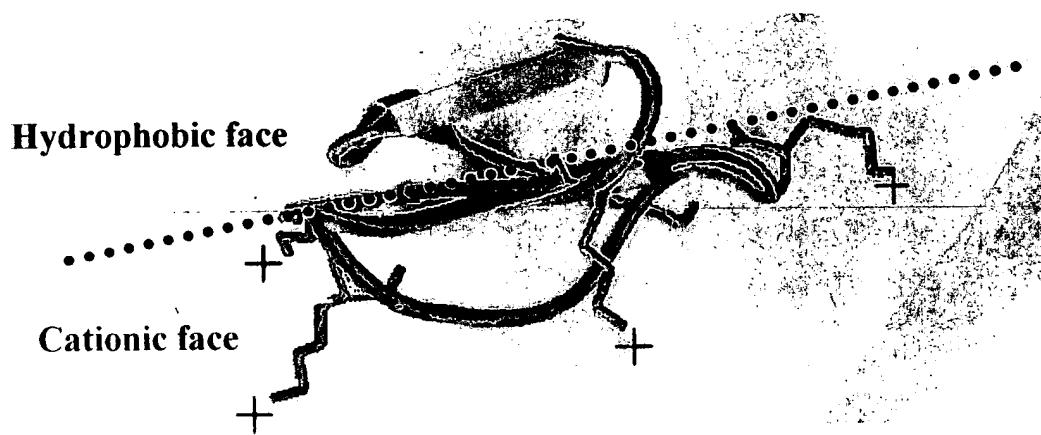


FIG.11

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Applicant(s): Rene Gantier, et. al

RATIONAL DIRECTED PROTEIN EVOLUTION USING TWO-DIMENSIONAL RATIONAL MUTAGENESIS SCANNING

2-D matrix representation of a protein sequence

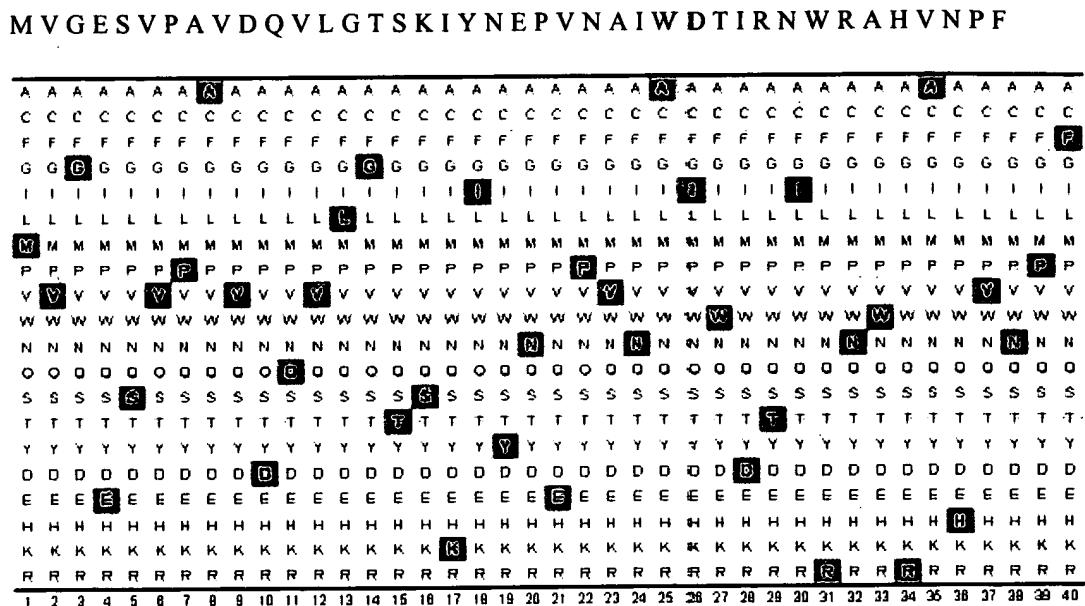


FIG.12

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Applicant(s): Rene Gantier, et. al

RATIONAL DIRECTED PROTEIN EVOLUTION USING TWO-DIMENSIONAL RATIONAL MUTAGENESIS SCANNING

2-D matrix for amphipathic peptide showing K/R scanning: sequential replacement of each residue by either K or R

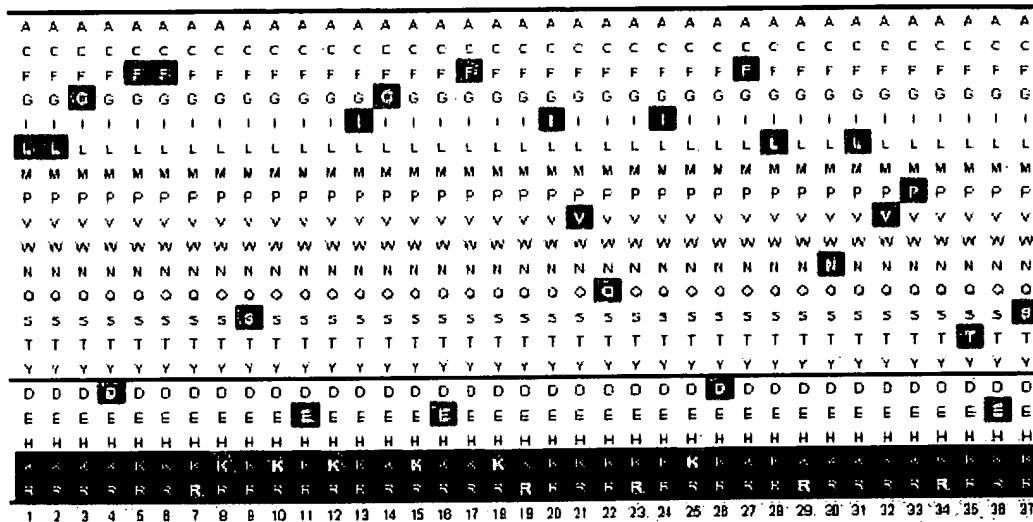


FIG.13A

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Applicant(s): Rene Gantier, et. al

RATIONAL DIRECTED PROTEIN EVOLUTION USING TWO-DIMENSIONAL RATIONAL MUTAGENESIS SCANNING

2-D matrix for K/R scanning on amphipathic polypeptide

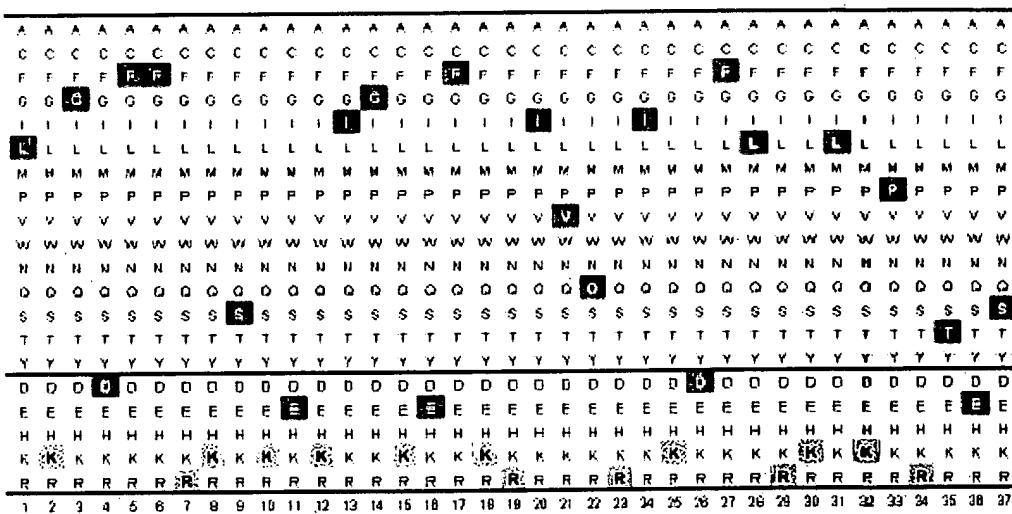


FIG.13B

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Applicant(s): Rene Gantier, et. al

RATIONAL DIRECTED PROTEIN EVOLUTION USING TWO-DIMENSIONAL RATIONAL MUTAGENESIS SCANNING

2-D matrix for LEAD candidates on amphipathic polypeptide

A large grid of letters and numbers from a crossword puzzle. The grid consists of 30 columns and 30 rows. The letters are arranged in a repeating pattern of black and white squares. Some letters are bolded, and some are in a different font style. There are also some numbers and symbols scattered throughout the grid.

FIG.13C

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Applicant(s): Rene Gantier, et. al

RATIONAL DIRECTED PROTEIN EVOLUTION USING TWO-DIMENSIONAL RATIONAL MUTAGENESIS SCANNING

2-D matrix for optimized amphipathic polypeptide, following both: i) K/R scanning (FIG. 13B) and ii) mutagenesis (FIG. 13C)

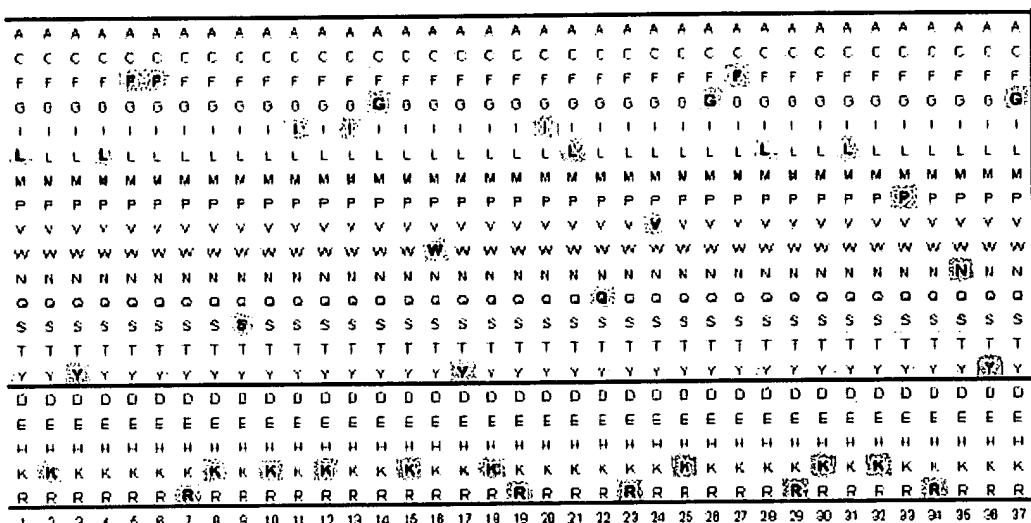


FIG.13D

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Applicant(s): Rene Gantier, et. al

RATIONAL DIRECTED PROTEIN EVOLUTION USING TWO-DIMENSIONAL RATIONAL MUTAGENESIS SCANNING

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Multi overlapped primer extensions for rational recombination of LEADS

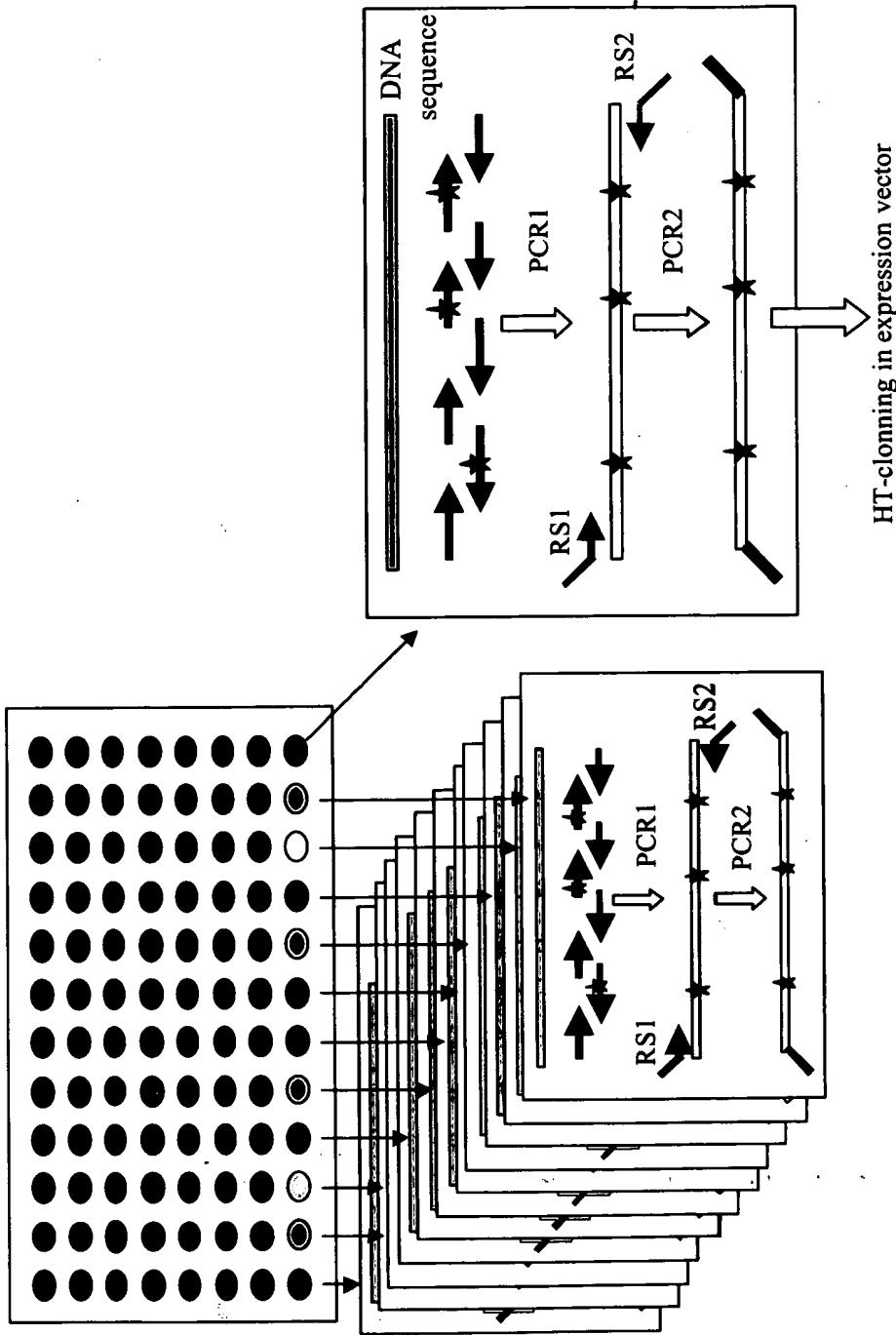


FIG. 14